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Isolation and Identification of Heavy-metal Resistant Soil Bacteria

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Abstract: Normal alluvial (FFS), calcareous (WNS) as well as sewage farm (ARS) soil samples amended with peptone nutrient solution were subjected to increasing concentrations of Pb, Cd, Zn and Ni then incubated for 7 days at 25°C. Total bacteria recorded the highest colony forming unites ($cfug^{-1}$) in Abu-Rawash soils (ARS) even in the presence of increasing levels of Cd, Zn and Pb followed by the normal alluvial (FFS) and calcareous (Wadi- El- Natrun) soils. Under Ni stress, calcareous soil showed the highest population density which may be due to the lime effect. In alluvial soil, counts were substantially reduced to 1% compared to plain soil applying only 3 mg Cd/kg soil while it was at 10% in Abu-Rawash soil. For Pb- and Zn-contaminated soils, microbial density in alluviall soil recorded almost the same density as in Abu-Rawash and was higher than in calcareous soil. Dehyrogenase activity (DEH) decreased by 32% in the FFS, 35% in ARS and 17% in WNS as the applied Cd increased from 3 to 8 mg/kg soil. Increased amounts of Zn and Ni inhibited dehydrogenase (DEH) activity by higher 36-44% in FFS and ARS compared to that of WNS (17-22%). A significant positive correlation coefficient ($P<0.05$) was observed between microbial counts and DEH activity in almost all examined heavy metal-polluted soil. Sixty-seven bacterial isolates from the three heavy-metal-contaminated soils were *in vitro* examined for their ability to tolerate increasing concentrations of Cd, Zn, Pd and Ni in their culture media. A screening of 67 bacterial isolates was conducted on solid nutrient agar medium supplemented with increasing concentrations of the investigated metals. Nineteen isolates were selected according to their heavy metal tolerance. Some isolates were found to be able to grow in a medium containing up to 700 mg Pb/kg others were tolerant to 75 mg Zn/kg. A maximum tolerable level of 25 mg Ni/kg soil was recorded with the isolates Nos. B-1, B-24, B+27, B-30, B+41, B+55 and B+64. The cultural, morphological and physiological characteristics of the heavy metal-resistant isolates were examined. The majority were Gram-positive long rod-shaped motile sporeforming isolates belonging to the genus *Bacillus*. The minimum inhibitory concentrations (MIC) of the examined heavy metals were designated for these isolates in submerged liquid cultures. For all examined isolates, culture OD_{600} increased with time reaching a maximum after 10 day and declined thereafter. Among the examined isolates, the two Gram-positive *Bacillus* isolates Nos. B-48 and B+41 were the most tolerant to all heavy metals. The identities of these isolates were analyzed using the Sole-Carbon Cource Utilization Profile (Biolog, Inc., Hayward, CA2000). The isolate No. B-48 was identified as *Bacillus amyloliquefacien*s and No. B+41 as *Bacillus thuringiensis*.

Key words: Bacterial resistance · Heavy metals · Dehydrogenase activity · Sewage farm soil

INTRODUCTION

Urban soils have been affected by elevated levels of heavy metals since the beginning of industrialization. In many situations, these heavy metals pose a considerable threat to the environment. Soil quality criteria are presently depends on total metal concentrations in soil. The environmental stress caused by heavy metals, generally, decreases the diversity and activity of soil bacterial populations leading to a reduction of the total microbial biomass, decrease in numbers of specific populations such as and a shift in microbial community

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structure [1]. Soil microbial population responses to heavy metal contamination provide a relevant model for ecological studies to assess the influence of environmental characteristics [2].

Various microorganisms show a different response to toxic heavy metal ions that confer them with a range of metal tolerance [3]. Several studies have demonstrated that metals influence microorganisms by affecting their growth, morphology and biochemical activity [4, 5] and diversity [6]. The response of the bacterial populations to heavy metal contamination depends on the concentration and bioavailability of the metal itself and is dependent by multiple factors such as the type of metal and microbial species [7]. Heavy metal contaminated environments harbor organisms, both prokaryotes and eukaryotes, able to deal with pollution [8]. The ability of some microorganisms to tolerate heavy metals and the ability of some to promote transformations that render them less toxic, make organisms that live in heavy metal contaminated sites potentially useful in bioremediation. High concentrations of metals (both essential and non-essential) harm the cells by displacing the enzyme metal ions, competing with structurally related non-metals in cell reactions and also blocking functional groups in the cell bio-molecules [9].

Studies on the effects of metals on soil microbiome have been conducted showing that short term contact causes the selection of resistant bacteria within weeks. A more prolonged exposure to metals slowly selects resistant bacteria. On the other hand, long term exposure to metals leads to the selection/adaptation of the microbial community which then thrives in polluted soils [5, 10]. The presence of different metals together may also have greater adverse effects on the soil microbial biomass/activity and diversity than those caused by single metals at high concentrations [11].

The aim of this study was to investigate the diversity, the metal tolerance and identification of tolerant bacterial species or strains present in different soil types heavely contaminated with Cd, Pb, Zn and Ni metals. Moreover, heavy metal tolerance of the isolated populations was also tested.

MATERIALS AND METHODS

Three topsoil (0-20 cm depth) samples were collected to represent alluvial soil (FFS) from the Experimental Station of Faculty of Agriculture, Cairo University, Giza, calcareous soil from Wadi-El-Natrun (WAS) and a sewage farm sandy soil from Abu-Rawash (ARS). The collected soil samples were air-dried, crushed and stored as fine earth (< 2mm). Samples were analyzed for pH and EC in 1:2.5 soil: water extract where soluble cations and anions were measured according to McLean [12]. Particle size distribution was assessed by the pipette method adopted by Gee and Bauder [13] while organic matter by wet oxidation method and total carbonate as described by Sparks [14]. Aqua-regia extractable heavy metals [15] and those extracted with DTPA (1: 2 soil: extractant ratio) were performed according to Lindsay and Norvell [16] and ISO 14870 [17]. Physical and chemical soil characteristics are summarized in Table 1.

Portions of 100 g from each soil type were weighed in 150 ml plastic cups and artificially contaminated with increasing concentrations of Cd (0,3,5 and 8 mg/kg), both Pb and Zn (70,250 and 400 mg/kg) and Ni (50,100 and 400 mg/kg) as sulfate solutions. Ten ml aliquots of 1% peptone/water solution were added to each soil cup which was further watered up to the field capacity (F.C.) using distilled water then incubated for 30 days at 25°C with maintaining the F.C. moisture level throughout the incubation period. For each soil type, peptone-amended soil cups without heavy metals were included for comparison. At the end of the incubation period, soils were sampled and analyzed for DTPA extractable Cd, Zn, Pb and Ni besides total plate bacterial counts.

Enumeration, Isolation and Identification of Heavy-metal Resistant Soil Bacteria: The bacterial densities were counted in soil samples serially diluted in 0.5% NaCl and plated onto nutrient agar medium [18]. Plates were incubated at 25 °C for 10 days where the colony forming unites ($cfug^{-1}$) were enumerated [19].Well-separated colonies with different morphological features were picked on nutrient agar slants then the purity of these isolates was checked by Gram staining with repeated streaking on nutrient agar plates. A total of 67 bacterial candidates was isolated from the three soil types contaminated with increasing heavy metal concentrations. Bacterial isolates were maintained throughout the work in nutrient broth culture containing 15 % (v/v) glycerol at 4.0°C [20]. The identity of the isolates was primarily designated relying on some morphological, cultural and biochemical traits described in Bergy's Manual of Determinative Bacteriology [21]. The isolates were conclusively identified adopting a Sole-Carbon-Source-Utilization Profile analysis using a BIOLOG GP2 MicroPlate™ Gram - positive Identification Test Panel (Biolog, Inc., Hayward, CA 2000).

Table 1: Physical and chemical characteristics of the investigated soil types

Soil Analysis			Faculty's Farm (FFS)	Abo Rawash (ARS)	Wadi El-Natrun (WNS)
		C.S.	3.5	20.5	24
Particle size distribution		F.S.	13.5	34.5	32.3
%		Silt	23	25.5	27.9
		Clay	60	19.5	15.8
		Class	clay	Sandy loam	Sandy loam
pH (1:2.5)		7.76	7.32	8.06	
EC dS/ m (1:2.5)		1.43	1	1.26	
Soluble anions (me/l) (1:2.5)		HCO_3	3.3	4.1	2.9
		Cl^-	4	2.6	3.8
		$SO^{=}_4$	7.5	3.6	6.6
Soluble cations (me/l)(1:2.5)		Na^+	7	3.1	5.5
		K^+	0.3	0.8	0.2
		Ca^{++}	5.4	4.3	6.4
		Mg^{++}	2.2	2.1	1.2
O.M		%	1.3	6.1	0.8
$CaCO_3$		%	1.1	2.6	14.4
Total N		%	0.14	0.24	0.08
Heavy metals (mg/kg soil)	Pb	AQ.R*	2.75	133.15	10.2
		DTPA	0.76	6.63	1.2
	Cd	AQ.R	2.75	0.9	0.06
		DTPA	0.03	0.14	0.02
	Zn	AQ.R	77.8	446.6	55.1
		DTPA	5.5	23.06	7.6
	Ni	AQ.R	4.03	2.23	0.48
		DTPA	0.55	0.1	0.05

AQ.R*= Aqua-Regia

extract Metal levels	Cd	Pb	Zn	Ni
1	3	100	70	50
2	5	250	250	100
3	8	400	400	400

Screening the Bacterial Isolates for Heavy Metal Resistance: Bacterial isolates were *in vitro* examined for their heavy metal resistance on solid and in liquid cultures according to Bisht *et al*. [22]. A loopful of 24-old slant culture was inoculated into 5 ml sterile distilled water. After vortexing; 0.1 ml of the culture suspension was inoculated onto agar medium supplemented with increasing concentrations of the examined heavy metals at the following concentrations (mg/l):

Level	Cd	Zn	Pb	Ni
	A	B	C	D
1	2	25	100	25
2	5	50	300	50
3	8	75	500	75
4	11	100	700	100

Cultures were incubated at 30°C for 10 days then growth was visually described as follows: -, no growth; +, poor growth; ++, good growth and +++, exuberant growth. Some selected heavy metal resistant isolates were further challenged for minimum inhibitory concentrations (MIC) of the examined heavy metals. Growth of the isolates was monitored in 250-ml Erlenmeyer flasks containing 50 ml nutrient broth supplemented with the highest tolerable concentrations of heavy metals resulted from the abovementioned screening tests. Submerged growth was followed by measuring the optical density at 600nm (OD_{600}). Cultures were incubated on a shaker incubator at 30°C for 10 days. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) [23].

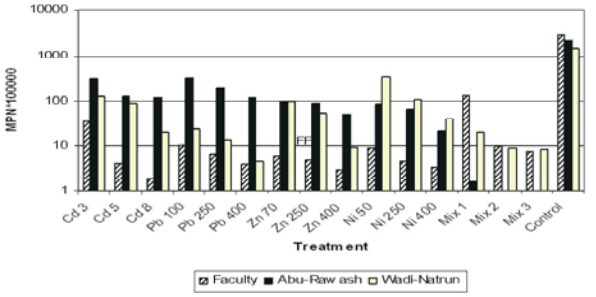
Dehydrogenase Activity in Soil: Dehydrogenase activity was determined in the soils as described by Casida *et al*. [24] and Blidar *et al*. [25] with the following modification: 1 g soil was mixed with 0.01 g of $CaCO_3$ incubated with 1 ml 3% 2, 3, 5 tri-phenyl tetrazolium chloride (TTC) and 3 ml water at 37 °C in darkness. After 6 h, 10 ml methanol were added and the suspension was homogenized, filtered and washed with methanol until the reddish colour caused by the reduced TTC (triphenyl formazan,TPF) had disappeared from soil. The optical density was measured at 485 nm in comparison to those of triphenyl formazan (TPF) standards. Results of enzyme activities are presented on oven-dry soil weight basis.

RESULTS AND DISCUSSION

The total bacterial counts in the three investigated soils spiked with increasing levels of Pb, Cd, Zn and Ni are presented in Fig.1. The highest count was recorded in Abu-Rawash (ARS) soil even in the presence of increasing levels of Cd, Zn and Pb followed by the normal alluvial (FFS) and calcareous (WNS) soils. Abu-Rawash soil (ARS) which is known to be enriched for long time with organic wastes sourced from the sewage sludge effluents. This might enhance building up high microbial populations in such soil type which might explain the superiority of this soil type in its microbial population density over other examined soils. In this regard, Kellya *et al.* [26] stated that sludge-amended soils exhibit a significant increase in counts of culturable bacteria.

Under stresses of Ni, Wadi- El-Natrun soil (WNS) recorded the highest bacterial density followed by Abu-Rawash (ARS) and the FFS soils. Most probably the high lime content of Wadi-Natrun soils reduces the toxic effect of Ni ions on the microbial populations in such soil type. McNear *et al*. [27] found that in crop plants, high soil Ca reduces Ni uptake and phytotoxicity and so it is probably has a similar effect on soil microbial populations. Moreover, the relatively high pH of Wadi-EL-Natrun calcareous soils might alter the applied Ni speciation into less toxic forms. These results corroborate the findings reported by Kukier and Chaney [28] referring to a similar reduction in available-Ni with increasing pH due to liming.

Fig. 1 shows decreased densities of microorganisms in different magnitudes due to increasing the concentration of the applied heavy metals in all tested soil types. Microbial populations inhabit alluvial soil (FFS) might be more sensitive to stresses exerted by the investigated heavy metals and thus the counts conspicuously reduced to nearly 1% of the natural density by applying only 3 mg Cd/kg soil while it was at 10% in Abu-Rawash (ARS) soil. In addition, increasing Cd in alluvial soil (FFS) from 3 to 8 mg/kg resulted in decreased bacterial counts by 95% while reductions of 83 and 61% for Wadi-El-Natrun (WNS) and Abu-Rawash (ARS) soils. Were recorded Probably the indigenous microbial populations in Abu-Rawash (ARS) soil are relatively more adapted to high level of Cd toxicity than in alluvial (FFS) one. Regarding Pb and Zn- contaminated soils, population densities could be arranged in an order FFS = ARS > WNS, whereas in Ni-contaminated the order soil population densities was (FFS) > (ARS) > (WNS).



Metal levels	Cd	Pb	Zn	Ni
1	3	100	70	50
2	5	250	250	100
3	8	400	400	400

Fig. 1: Total plate counts (cfug^{-1}) of soil bacteria in different soil types as affected bydifferent concentration of heavy metals (mg/kg)

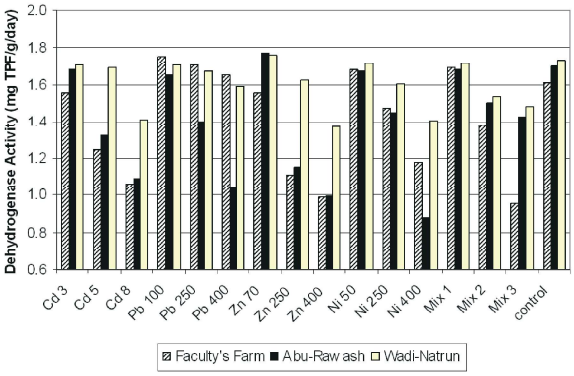


Fig. 2: Dehydrogenase activity of the three soil types as affected by increased applied rates of Cd, Pb, Zn and Ni.

A surprising effect was observed with the heavy metal mixture, where the highest microbial count was recorded in the alluvial (FFS) soil contaminated with a heavy metal mixture (Cd1+Pb1+Zn1+Ni1) and even at higher combination levels while didn't show up in Abu-Rawash (ARS) soil. However, data show that the presence of metal combinations may have adverse effects on soil microbial biomass and diversity than those caused by single metals at high concentrations which is in agreement with Renella *et al*. [11].

Dehydrogenase (DEH) activity reflects the total oxidative activity of the microbial biomass. Mean values of DEH activity were significantly lowered due to increased heavy metal levels in the amended soils (Fig. 2). The inhibition rate of DEH activity was significantly higher in FFS sample and ARS than that in WNS.

Table 2: Regression between bacterial MPN and dehydrogenase activity in the investigated soils spiked with Cd, Pb, Zn and Ni metals

		FFS	ARS	WNS
Cd	R	0.8858*	0.6461	0.8765*
	slope	0.1735	0.1942	0.3436
Pb	R	0.8690*	0.8441*	0.7966
	Slope	0.0497	0.1058	0.2434
Zn	R	0.8345*	0.7687	0.8690*
	slope	0.2406	0.2412	0.3929
Ni	R	0.7635	0.8001	0.8978*
	slope	0.1729	0.1548	0.5428

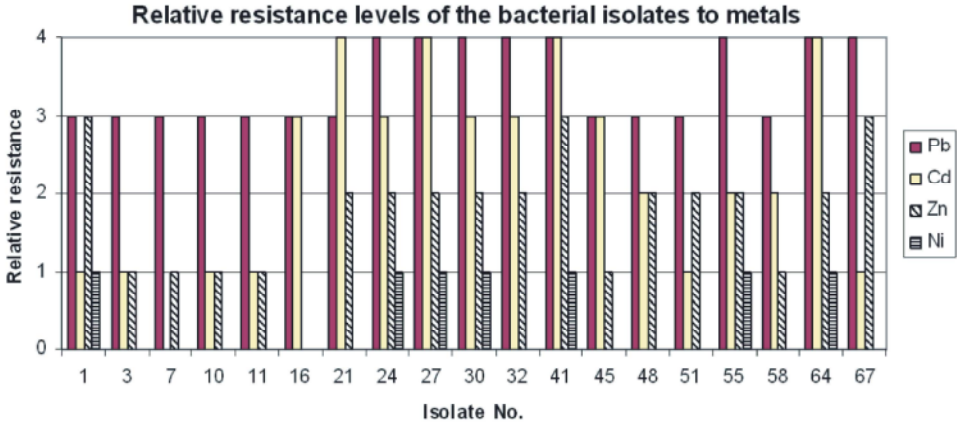
Relative Resistance	Pb	Cd	Zn	Ni
1	100	2	25	25
2	300	5	50	50
3	500	8	75	75
4	700	11	100	100

Dehyrogenase activity decreased by 32% in FFS, 35% in ARS and 17% in WNS as the applied Cd increased from 3 to 8 mg/kg soil. Increasing Zn and Ni also inhibited dehydrogenase activity by higher percentages (36-44%) in FFS and ARS compared to WNS (17-22%). The presence of relatively higher $CaCO_3$ content (144 g/kg) in Wadi-El-Natrun soil and its corresponding high pH of 8.05 might help mitigate heavy metal toxicity which may adopt the production of dehydrogenase enzyme. Mijangos *et al*. [29] stated that soil liming significantly increased dehydrogenase activity (as an indicator of soil microbial activity) which related to the increased soil pH.

There is a significant positive correlation coefficient ($P<0.05$) between bacterial counts (Table 2) and dehydrogenase activity. As the microbial count increased, dehydrogenase activity increased with different rates depending on soil type. In Wadi-El-Natrun soil (WNS) the slope of dehydrogenase / log count was much higher than that of Faculty's farm soil (FFS) in all the tested metals. This indicates the positive effect of high bacterial density on dehydrogenase activity in calcareous soil as compared to alluvial one which conform data reported by Mijangos *et al*. [29].

Heavy metal applications significantly inhibited the soil DEH activity which is in harmony with previous reports of Marzadori *et al*. [30] and Garc´ýa-Gil *et al*. [31] who found that DEH activity was retarded toxic effect of heavy metals added to the soil. Sardar *et al*. [32] stated that DEH activity appeared to be one of the most sensitive parameters among the selected enzyme activities and it could be used as a good indicator of soil ecotoxicological test caused by heavy metals.

A screening was conducted using 67 bacterial isolates on solid nutrient agar media supplemented with increasing concentrations of the investigated metals. For each isolate, the maximum tolerable level of each metal was recorded. Fig. 3 shows that bacterial isolates were screened to only 19 heavy-metal tolerant ones. It was found that some of the screened isolates were tolerant to up to 700 mg Pb/kg (*e.g.*isolates Nos. B-24, B+27, B-30, B+41, B+55, B+64 and B+67), while only four isolates (Nos. B-21, B+27, B+41 and B+64) tolerated a maximum Cd



Relative Resistance	Pb	Cd	Zn	Ni
1	100	2	25	25
2	300	5	50	50
3	500	8	75	75
4	700	11	100	100

Fig. 3: The relative resistance of the bacterial isolates for the maximum tolerance heavy metal concentrations

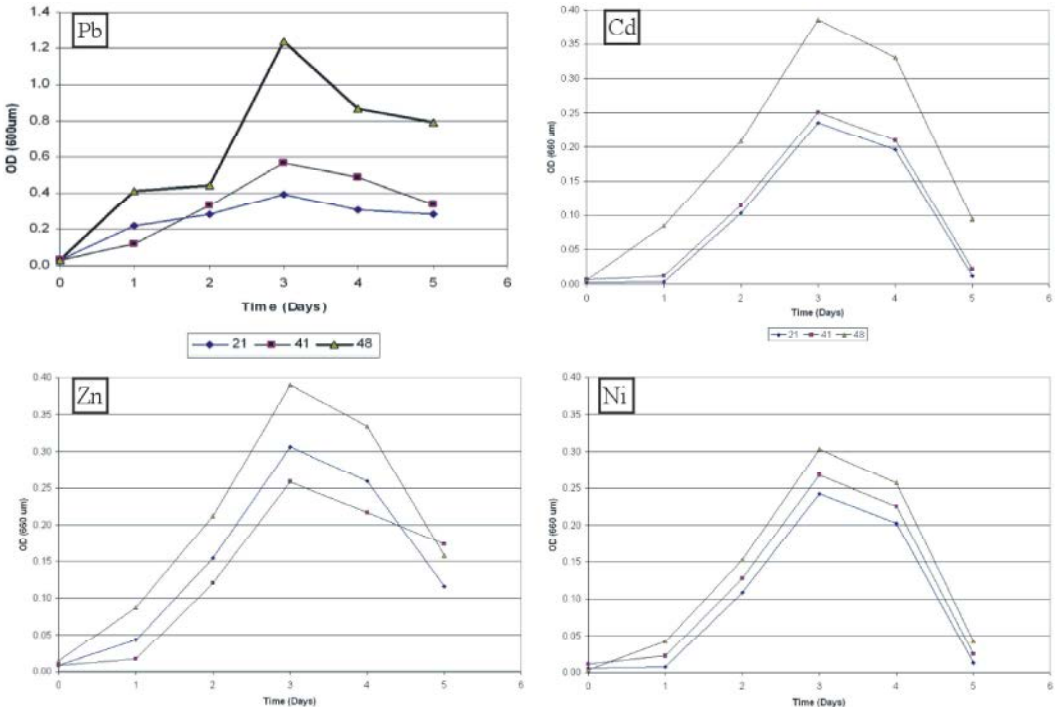


Fig. 4: Growth curve of the most tolerant isolates (B-21, B+41 and B-48)

concentration of up to 11mg Cd /kg. Three isolates *i.e.*No.B-1, B+41 and B+67 were tolerant to 75 mg Zn/kg, while the maximum tolerable level of Ni (25 mg/kg) was recorded for isolates Nos. B-1, B-24, B+27, B-30, B+41, B+55 and B+64.

The screened isolates were grown in liquid nutrient medium amended with the previously designated metal concentrations. Growth of these selected isolates was monitored by measuring the culture turbidity (optical density, OD_{600}) and the results are illustrated in Fig.4. All the tested isolates showed different responses towards high metal stresses. The optical density increased with increasing incubation period and the increases were different according to the stress of heavy metal which reached its maximum after 3 days and declined thereafter in all treatments. Despite the increased concentrations of the examined heavy metal in the growth medium, a sustainable growth was exhibited by the isolates B-48 and B+41. Their morphology showed that they are G-positive, long-rode-shaped, motile sporeforming Bacilli. Cell wall constituents have an important role affecting the immobilization of heavy metals [33].

A conclusive identification of the most tolerant isolates B-41 and B-48 was applied adopting a Sole-Carbon-Source-Utilization Profile analysis using a BIOLOG GP2 MicroPlate™ Gram - positive Identification Test Panel (Biolog, Inc., Hayward, CA 2000). The obtained results affiliated isolate No. B-48 to *Bacillus amyloliquefacien*s and isolate No. B+41 to *Bacillus thurengensis*. In this context, Rathnayake *et al* [34] demonstrated a potent heavy metal resistance by Gram-positive sporeforming motile rode-shaped soil bacteria and identified two *Bacillus* strains as *B. thuringiensis* and *Paenibacillus* sp. the most Cd and As-resistant soil microorganism was identified as *Bacillus subtilis* by Ali *et al*. [35].

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